Balarabe *et al.* (2022)
Biological and Environmental Sciences Journal for the Tropics 19(3) December, 2022
ISSN 0794 – 9057; eISSN 2645 – 3142

BEST JOURNAL 19(3): 53 - 63
Date received: 21/12/2022
Date accepted: 24/12/2022
Printed in Nigeria

SINGLE NUCLEOTIDES SUBSTITUTION FOR THE FORMATION OF TWO-SITE MUTAGENIC *HGGT* cDNA GENE IN *Elaeis guineensis* BY PCR OVERLAPS EXTENSION

Sulaiman Rufa' I Babura
Department of Plant Biology, Faculty of Life Sciences, Bayero University, Kano Nigeria
srbabura.bot@buk.edu.ng; Phone no. 08069588538

ABSTRACT
Polymerase Chain Reaction has become an easy and quick method to use in the alteration of any gene sequence, this is because single or multiple sequences changes can readily be incorporated chemically into oligonucleotide primers to produce required modification. A method of adding two mutations in Homogentisate Geranylgeranyl Transferate (HGGT) cDNA gene of *Elaeis guineensis* was carried out to generate three (3) different cDNA sequences molecules that are 99.99% homologous to original wild type cDNA (DxP HGGT). This is by simple and flexible method of site-directed mutagenesis driven by two step PCR overlap extension. Single nucleotide mutagenesis was successfully carried out to generate three cDNA sequence variants (193SNPHGGT, 2429SNPHGGT and 2SNPHGGT) with one or both SNP variants incorporated into the sequence of the commercial D×P genotype HGGT gene. The first PCR produces two overlapping fragments with introduced nucleotide substitution at 101 bp and 1288 bp for the first mutation, 629 bp and 760 bp for the second mutation from the full length cDNA of 1389 bp. The intermediate fragments were hybridized at their 3'end in the subsequent PCR and full length cDNA of 1389 bp were amplified by outer primers which are modified to includes gateway recombination properties to ligate the products into expression vector pB7WG2D,1 for subsequent molecular studies. Coding regions of *Elaeis guineensis* HGGT gene was successfully mutated by site-directed mutagenesis and amplified using PCR overlap extension without necessarily cloning the gene of interest into any plasmid before been use for mutagenesis.

Keywords: Mutagenesis, Sequence variants, HGGT gene, PCR overlaps, Primers

INTRODUCTION
Gene sequence can be altered by insertion, deletion or substitution through the use of oligonucleotide primers in a PCR reaction to meet a specific need (Heckman & Pease, 2007). PCR has become an easy and quick method to use in the introduction of any gene sequence changes when compared with other recombinant techniques (Kiryu et al., 2014) this is because single or multiple sequences changes can readily be incorporated chemically into oligonucleotide primers to produce required modification (Kaur & Sharma, 2006). Introducing mutagenesis by overlap extension into gene segments has been described as a method to create either base substitutions, insertions or deletions by site-directed substitution (Urban, 1997). The genomic DNA or cDNA of a gene is firstly denatured thermally to produce single strands, which followed by the second step of annealing with a primers that bears the mutations. In the third step, primer extension is accomplished by high fidelity Taq polymerase enzymes with proof-reading ability. (McCullum *et al.,* 2010)

The structural and functional relationship of a genes and proteins led to the emergence of both single and multiple site-directed mutagenesis (Peng, et al., 2012). The method is quite simple and efficient especially if the initial materials are critically selected and primers design is carefully done (Chiu *et al,* 2004).

The target gene is amplified with two flanking outer primers design at 5'ends of the gene and complimentary internal forward and reverse primers that introduce the mutation (Liu, et al, 2012). The final product of this experiment was inserted into an expression vector according to Gateway cloning technology. This cloning technology is a recombination method that cloned gene of interest into an entry vector without following traditional restriction enzyme digestion and ligation method. The main aim of Gateway cloning is to generate an expression clone. It involves two step processes; first is the cloning of gene of interest amplified by joint attB and specific primers into the entry vector followed by subcloning of the gene from entry clone into a destination vector using the LR Reaction protocol to produce the Expression Clone.(Hilson et al., 2006)

In this study full length HGGT cDNA sequence was isolated from the 19 week after antheasis. *E. guineensis* mesocarp tissues and the sequence exhibited high similarity with the previous known sequences of HGGT from different plants. It is manifested to be sequence of low vitamin E content accession. In order to ascertain the response of this gene sequence in vitamin E production, the cDNA was cloned into destination vector by gateway cloning together with three (3) different mutated HGGT cDNA sequences (*193M HGGT, 2429M HGGT, 2M HGGT*) that was produced through site-directed mutagenesis to look similar with high vitamin E HGGT sequences.

MATERIALS AND METHODS

Plant Materials, Total RNA Extraction and RT-PCR

Mesocarp tissue of 19-20 w.a.a of oil palm (*Elaeis guineensis* var. Dura x Pisifera) fruit was obtained from UPM oil palm plantation field. Total RNA was extracted from 3 g of mesocarp tissue by using a modified CTAB method (Presccott and Martin, 1987). About 1 µg of total RNA was treated with *DNase I,* RNase-free enzyme (Thermo SCIENTIFIC) as recommended by the manufacturer. Total RNA of (~1 µg) was reverse transcribed to create the first strand cDNA, using SuperScript® III first strand synthesis system kit (Life Technologies-Invitrogen) according to the manufacturer's protocol. About 2 µl of the first strand reaction mixture was used for normal PCR reaction (Bio-Rad), directly. Gene specific primers: forward primer $^{5'}$ATGGCAAAAGAAGAGTTTATC$^{3'}$ and reverse primer $^{5'}$CCTTATCCCTTTCATGCACTG$^{3'}$ were used to isolate complete sequence of HGGT coding region (GenBank accession No. KP878511). The PCR condition was set at initial denaturation for 3 min at 95 °C followed by amplification for 35 cycles at 98 °C for 20 sec, 60 °C for 15 sec and the extension temperature of 72 °C for 2 min then followed by a final extension at 72 °C for 5 min.

Site-directed Mutagenesis Primers Design

The two outer flanking forward and reverse primers of 21 nucleotides each were used to amplify end to end cDNA sequence of the whole *HGGT* gene. 5'end of forward primer (A) started from ATG start codon, while reverse primer (D) was designed to start from stop codon. The two internal reverse (B) and forward (C) primers were designed with the mutated site and are completely complementary with one another. The base substitution for mutation is located between 5 to 10 nucleotide from 5'end of the forward primer. The primers were designed with all the primer design parameters such as melting temperature (Tm) between 60°C-70°C, GC content which is between 40%-60% (optimum 50%) taken into consideration. To create mutagenesis at 91 base location, cytosine (C) is replace with thymine (T) at base 10 of forward internal primer, while guanine (G) is replace with adenine (A) at position 11 of the reverse internal primer.

The mutation at position 615 was guanine (G) is replace with adenine (A) at position 6 of forward primer, while cytosine (C) is replace with thymine (T) at position 15 of the reverse primer (Table 1)

Primary (1st) PCR Reaction

Two PCR products (AB and CD) fragments was produce using primers A and B for AB fragment and primers C and D for CD fragment. The PCR reaction was runs at a final volume of 25 µl by mixing [5 µl of 5X KAPA HiFi buffer (high fidelity), 1.5 µl of 10 mM KAPA dNTP mix, 1.5 µl of each 10 µM forward and reverse primers, 1.0µl of 10mM KAPA HiFi Hotstart and 1.5 µl of cDNA (50 ng/µl) as template] in a 13µl nuclease free water. The reaction was heated as follows: Initial denaturation for 5 min at 95 °C followed by amplification for 35 cycles at 980C for 20 sec and annealing temperature 600 for 15 sec and the extension temperature of 720C for 1 min then followed by a final extension at 720C for 5 min. PCR product was run on 1% (w/v) agarose gel at 80 v for 80 min to confirm the amplification.

Secondary (2nd) PCR Reaction and Purification

The two PCR products AB and CD was used as template in this PCR reaction, with primer A and D as forward and reverse primers respectively. Equal concentration and quantities of AB and CD products was used regardless of their availability. A primary PCR reaction protocol was followed with only variation in annealing temperature. Negative control without AB and CD template was incorporated in separate tube to verify the reaction and/or contamination. Expected size of the second PCR product (AD) was excised from the agarose gel with a clean sharp scalpel and gel purified. Only the location of the desired band was excised to minimize the size of the gel and to avoid sequence overlap.

Sequencing and Mutagenesis Screening

Twenty (20) replicate of gel purified secondary PCR products (AD) was aliquot into separate tubes and send for sequencing with BGI sequencing Services Company Hong Kong Japan. The samples were sequenced using gene specific primers, forward: $^{5'}$ATGGCAAAAGAAGAGTTTATC$^{3'}$ and reverse: $^{5'}$CCTTATCCCTTTCATGCACTG$^{3'}$. The forward and reverse sequencing results of both the two mutations were aligned to each other to find any potential mutation error in the procedure using the Sequence Scanner ver. 1.0. Consensus sequence was deduced using the CLUSTAL W program from Bioedit ver. 7.2.5 software. The 1389 bp coding region was submitted to GenBank nucleotide blast database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for identity check and substituted mutation verification.

Gateway Cloning

Gateway Technology (Invitrogen) was used to clone the PCR products from cDNA. The PCR products was gel purified using QIAquick Gel purification Kit (QIAGEN) as recommended by the kit and used as template in another PCR reaction using primers designed base on gateway technology protocol: forward primer: 5´-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTA TAT GGC AAA AGA AGA GTT TAT CAA-3´; reverse primer with stop codon: 5´- GGG GAC CAC TTT GTA CAA GAA AGC TGG GTT TCA GTG CAT GAA AGG GAT AAG GA -3'. The PCR products were cloned in pDONR/zeo vector to create the Entry clone using Gateway® BP Clonase® II enzyme mix (Invitrogen; cat. No.: 11789-020). The plasmid was extracted and purified and subsequently cloned downstream of 2xT35S CaMV promoter into pB7WG2D,1 (Karimi and Depicker, 2002) as a destination vector using LR recombination reaction (Catalog no. 11791-020, Invitrogen) according to manufacturer's method.

RESULTS

Isolation of *EgHGGT* cDNA

The full length cDNA sequences of *EgHGGT* coding region were amplified from the total cDNA synthesized using gene specific primers. The expected band sizes were found when the PCR product was run in agarose gel. Sequence analysis revealed that *EgHGGT* has a coding region of 1389 bp which has 99% homology with other previously characterized Eg*HGGT* (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (Figure 1)

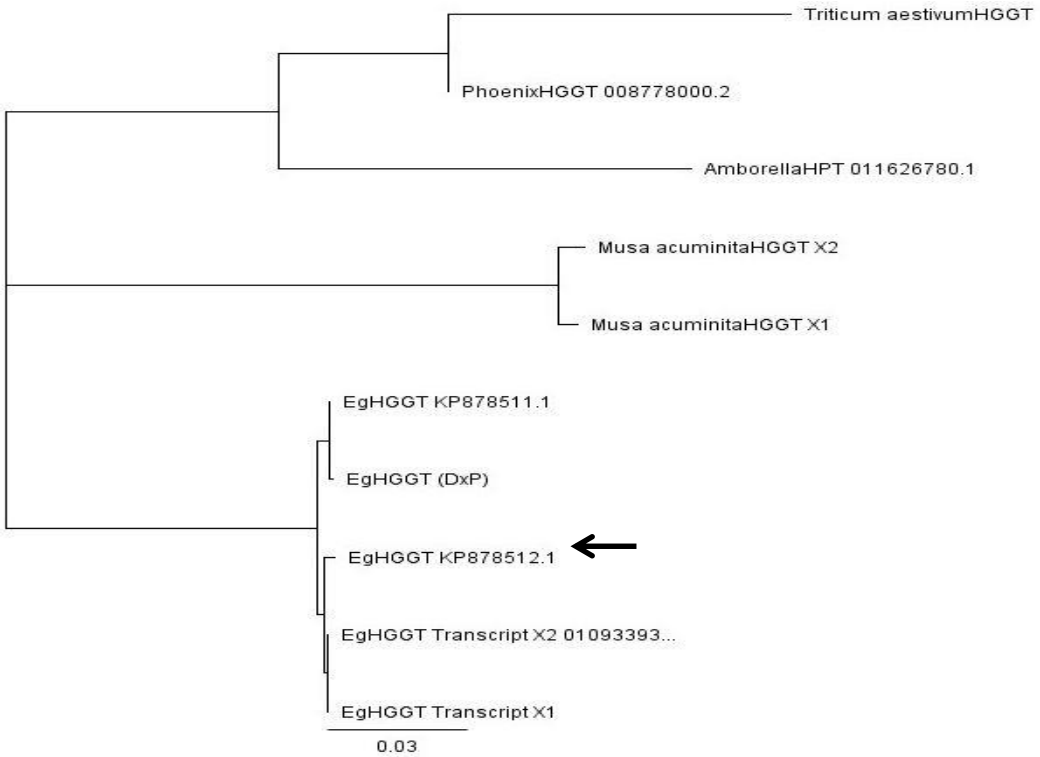

Figure 1: Phylogenetic analysis of HGGT cDNA from Elaeis guineensis DXP with other homologous HGGT cDNA constructed with Geneious 9.1.5 software. Arrow shows the commercial EgHGGT (DXP).

Site-directed Mutagenesis

Heckman and Pease, 2007 protocol was used to introduce the two (2) important nucleotide substitutions into the cDNA sequence of commercial D x P HGGT gene of 1389 bp. This substitutions lead to the change in amino acid residue from proline to serine at position 91 and methionine to isoleucine at position 615. Mutation at 91 was first produced by four pairs of primers (Table 1), two outer bound forward and reverse primers (a and d) that amplified the whole 1.389 kb sequence and two internal overlap primers (b and c) that introduce the mutation of interest at the SNPs location. The primary PCRs with primer pairs a + b and c + d produced cDNA fragments AB (101 bp long) (Figure 2a) and CD (1288 bp) (Figure 2b) respectively with primers b and c carrying the substituted nucleotides of AT instead of GC. The PCR products were clean up with PCR purification kit and the second PCR was run with primers a + d using equal concentrations (62 ng/µl) of AB and CD fragments as template to produce the complete cDNA segment (AD) (Figure 2c). There are multiple and unspecific band sizes in AD products when run in an agarose gel, this is due to amplification of differentially spliced site and that is always the case when cDNA was used as template (Heckman and Pease, 2007).

The correct product size was cut out and gel purified ignoring any other bands. Sequencing results of sixteen (16) AD products out of twenty (20) equivalents to (80%) (Table 2) shows successful changes of nucleotide from cytosine (C) to thymine (T) at position 91, which changes the encoded amino acid from Proline to Serine. The successful AD fragment of 1389 bp was named "*91M HGGT*".

The primary PCRs of the second mutation at 615 position produced cDNA segments AB (629 bp long) (Figure 2d) and CD (760 bp) (Figure 2e) with primers b and c carrying the substituted nucleotides of TA instead of CG. The two products were used as templates to generate AD products (Figure 2f). Sequencing results shows successful change of nucleotide in eighteen (18) AD products out of twenty (20) equivalents to (90%) (Table 2) from Guanine (G) to Adenine (A), which changes the encoded amino acid from Methionine to Isoleucine. The successful AD fragment of 1389 bp was named "*615M HGGT*".

The cDNA with the two introduced mutations was successfully produces by using one of the successful AD PCR products above as template. When *'91M HGGT'* was used as template, 615 primers was used to introduce the second mutation and when '*615M HGGT*' was used as template, 91 primers was used to perform both primary and secondary PCR. Sequence analysis shows that 60-100% (Table 2) AD fragments had incorporated the second mutation, which means the two mutation are successfully produced in one 1389 bp AD product and it is named as "*2M HGGT*"

Table 1: List of primers used in site-directed mutagenesis and Gateway cloning

Primers Name	Primers Sequence at 91 Position	Primers Sequence at 615 Position
Forward (A)	$^{5'}$ATGGCAAAAGAAGAGTTTATC$^{3'}$	
	G	C
Reverse (B)	$^{5'}$TTAGAGAGAGAAGAAGAAGA$^{3'}$	(B) $^{5'}$GCCTTCAATAGTCCTATAAA$^{3'}$
	C	G
Forward (C)	$^{5'}$TCTTCTTCTTCTCTCTCTAA$^{3'}$	(C) $^{5'}$TTTATAGGACTATTGAAGGC$^{3'}$
Reverse (D)	$^{5'}$CCTTATCCCTTTCATGCACTG$^{3'}$	
attB1-HGGT-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTATATGGCAAAAGAAGAGTTTATCAA	
attB2-HGGT-R	GGGGACCACTTTGTACAAGAAAGCTGGGTTTCAGTGCATGAAAGGGATAAGGA	

Table 2: Results of successful site-directed mutagenesis

Primers pairs	PCR fragments size (bp)	No. of PCR performed	% of successful mutation incorporated
AB1	101	40	26/40 (65%)
CD1	1288	40	29/40 (72.5%)
AD1	1389	20	16/20 (80%)
AB2	629	40	27/40 (67.5%)
CD2	760	40	33/40 (82.5%)
AD2	1389	20	18/20 (90%)
AD3	1389	40	34/40 (85%)

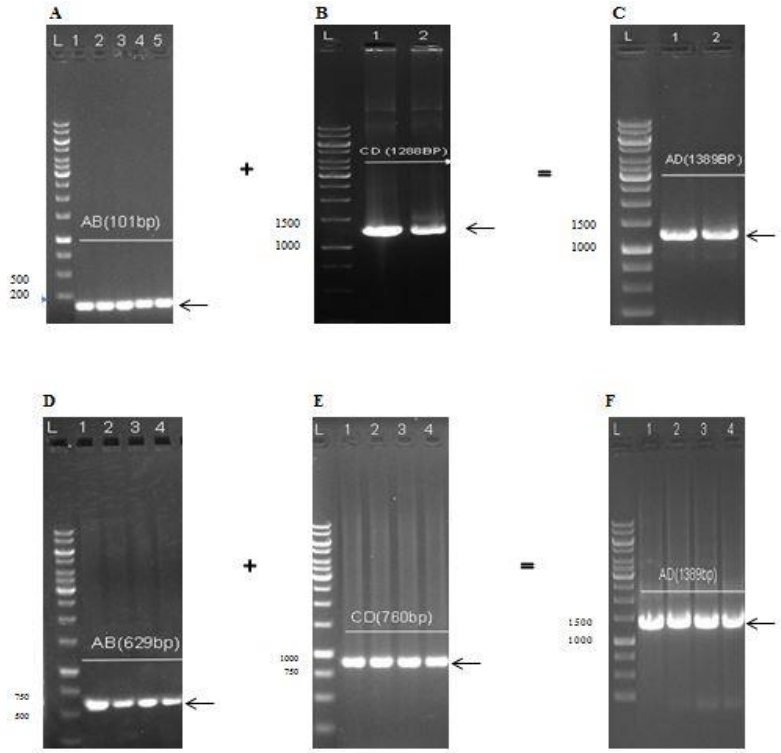

Figure 2: PCR products agarose gel electrophoresis analysis of site-directed mutagenesis of HGGT cDNA fragments (A) First Fragment AB of 101 bp (B) Second fragment CD of 1288 bp (C) Entire fragment AD of 1389 bp (D) First Fragment AB of 629 bp (E) Second fragment CD of 760 bp (F) Entire fragment AD of 1389 bp. Lane: L, Gene Ruler DNA ladder 1kb (ThermoScientific)

Gateway cloning

Three different set of 1389 bp cDNA HGGT sequences were developed as follows*: 91M HGGT*, *615M HGGT* and *HighM HGGT* from the original *Low HGGT* cDNA Sequence. All the four products was gel purified with minimal exposure to UV light and ligated into entry vector for gateway cloning using the manufacturer's instructions. *AttB*-set of gene specific primers (Table 2) amplified *91M HGGT, 615M HGGT, HighM HGGT* and *Low HGGT* from the purified PCR products using KAPA HiFi Hotstart PCR Kit. The purified *att*B-flanked products were cloned into pDONR^{TM}/Zeo in a recombination reaction using BP Clonase II enzyme and transformed into *E. coli*. Five (5) colonies of each 91 *HGGT, 615HGGT,* High *HGGT* and Low *HGGT* transformants picked for PCR screening with the *attB*-gene specific primers shows the expected insert size of ~1389bp. Plasmids were extracted from each confirmed colony (Figure 3a) and sent for sequencing with M13-flanking primers. All the entry clone plasmids were confirmed to have the correct full-length cDNA sequence with the important *attB1* and *attB2* recombination sites. Each confirmed plasmids were subsequently used in the LR recombination reaction with destination vector pB7WG2D1 to generate expression clones. Colony PCR was performed with 5 colonies of each transformants and the results shows the expected insert sizes (Figure 3c). Plasmids were extracted and sent for sequencing with gene specific primers (Figure 3b). Figures 5.13 show the sequences of confirmed expression clones which have the correct insert sequence with successful mutated sites.

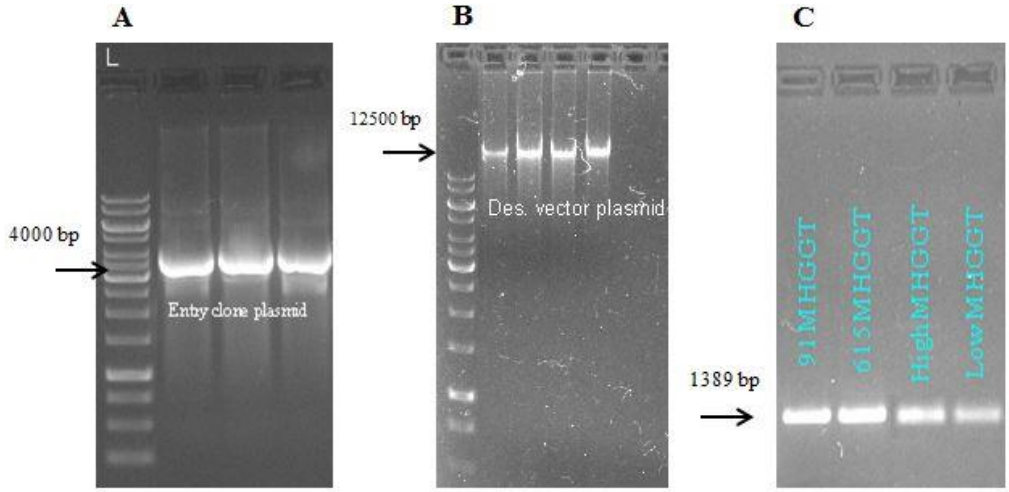

Figure 5.11: Gateway cloning of mutated HGGT cDNAs. (A) Entry clones plasmids, (B) Expression clones plasmids. (C) attB-flanked *193 HGGT*, *2429 HGGT*, *High HGGT* and *Low HGGT* PCR products amplified with KAPA HiFi HotStart PCR Kit.

Nucleotide sequences of expression clones from Gateway Cloning.

> pB7WG2D.1+ *91M HGGT*

START

TTTGTACAAAAAAGCAGGCTATATGGCAAAAGAAGAGTTTATCAATACAAATATTCGCAAATCA
GAATACTTCTCTCCTCTCCTCGCCGTGCTCCTCCTCTGTTCTTCTTCTCTCTCTCTAAATCAC
AATACTTCTCTTCTCTCCTCTCCTTGCCGTACTCCTCCTCTGTTCTTCATCTTCTCTCTTGGGA
AATGCTGTCCAACCAGGCTGTTTCAGTCTCCCGTTTTACCAAGTGTTGGCTCCCTCTAAGAGAT
GGGACAAGAGGAAGAGGGCCCATTACTGGTACTCAAGGGAGAGGTGGAATTTGGAAAGTAGAAA
ACTTGGAAGTCCAAGGAACAAGATGTAAATCCCCTAAATTCAAAGATATAGTCTATGATCTAAA
GCATAAAGAGAGGCCATCAATGCATTATAGACGACTTACAAAAGGTTGCATTTCTGCTGCTTCT
GAGCTTGCATATGTACCCGAACCAACGGATAATCAGTCACAAGGTCTATGGACTGCTGTTTCTA
AAAAGTTAGATGCTTTCTATCGGTTTTCTCGTCCCCACACAGTAATAGGCACTATCGTAGGCAT
AGTATCAGTCTCTCTTCTTCCAGTACAAAGTCTCGCTGATTTTTCTCCAACATATTTTATGGGA
CTATTGAAGGCATTGGTTCCAGCTGTATGCATGAACATCTATGTTGTGGGCCTGAACCAGCTAT
TTGACATAGAAATAGATAAGGTTAATAAACCTAGGCTTCCACTTGCTTCAGGCGAGTTCTCTTT
GGGAACAGGAATTGTGTTGGTATTTGCCGCTGCTATCATGAGCTTTACAATGGGGTTGCAGTCC
AAGTCCCCTCCCCTATTAGGTGCCTTGCTCATCAGCTGTTTTCTTGGAAGTGCATATTCCATCA
ATATTCCCTTCTTTAGATGGAAACAGCATGCCTTTCTTGCTGCATCTTGCATCTTGTGTGTGAG
GGCCGTTGTAGTCCAGCTAGCTTTTTTCATTCACATGCAGAGATATGTACTAGGAAGACCAACA
GTGTTGACAAAACCAGTGATATTTGCAACAGCTTTCATGTGTTTTTTCTCAGCTGTTATTGCTC
TGTTCAAGGATATACCTGATGTTGATGGAGACAGATATTTTGGCATCCAGTCCTTCAGTGTTCG
CCTGGGCCAAGAAAAAGTGTTTTGGTTTTGCATCAAGTTGTTGTTAACTGCATATGCTACAGCT
CTATTGGTGGGAGCTTCTACATCAACCATATATCAGAAGATTGTGACTGTGCTTGGCCATGGCT
TGCTTGCTTCCATTCTTTGGTTTCGGGCACAGTCTATTGATCTTCAGAACAAGGCATCCATAAC
CTCATTCTACATGTTCATTTGGAAGCTGTTTTATGCCGAGTATTTCCTTATCCCTTTCATGCAC
TGAAACCCAGCTTTCTTGTAC

STOP

> pB7WG2D.1+ *615M HGGT*

START

TTTGTACAAAAAAGCAGGCTATATGGCAAAAGAAGAGTTTATCAATACAAATATTCGCAAATCA
GAATACTTCTCTCCTCTCCTCGCCGTGCTCCTCCTCTGTTCTTCTTCTCCTCTCTCTAAATCAC
AATACTTCTCTTCTCTCCTCTCCTTGCCGTACTCCTCCTCTGTTCTTCATCTTCTCTCTTGGGA
AATGCTGTCCAACCAGGCTGTTTCAGTCTCCCGTTTTACCAAGTGTTGGCTCCCTCTAAGAGAT
GGGACAAGAGGAAGAGGGCCCATTACTGGTACTCAAGGGAGAGGTGGAATTTGGAAAGTAGAAA
ACTTGGAAGTCCAAGGAACAAGATGTAAATCCCCTAAATTCAAAGATATAGTCTATGATCTAAA
GCATAAAGAGAGGCCATCAATGCATTATAGACGACTTACAAAAGGTTGCATTTCTGCTGCTTCT
GAGCTTGCATATGTACCCGAACCAACGGATAATCAGTCACAAGGTCTATGGACTGCTGTTTCTA
AAAAGTTAGATGCTTTCTATCGGTTTTCTCGTCCCCACACAGTAATAGGCACTATCGTAGGCAT
AGTATCAGTCTCTCTTCTTCCAGTACAAAGTCTCGCTGATTTTTCTCCAACATATTTTATNGGA
CTATTGAAGGCATTGGTTCCAGCTGTATGCATGAACATCTATGTTGTGGGCCTGAACCAGCTAT
TTGACATAGAAATAGATAAGGTTAATAAACCTAGGCTTCCACTTGCTTCAGGCGAGTTCTCTTT
GGGAACAGGAATTGTGTTGGTATTTGCCGCTGCTATCATGAGCTTTACAATGGGGTTGCAGTCC
AAGTCCCCTCCCCTATTAGGTGCCTTGCTCATCAGCTGTTTTCTTGGAAGTGCATATTCCATCA
ATATTCCCTTCTTTAGATGGAAACAGCATGCCTTTCTTGCTGCATCTTGCATCTTGTGTGTGAG
GGCCGTTGTAGTCCAGCTAGCTTTTTTCATTCACATGCAGAGATATGTACTAGGAAGACCAACA
GTGTTGACAAAACCAGTGATATTTGCAACAGCTTTCATGTGTTTTTTCTCAGCTGTTATTGCTC
TGTTCAAGGATATACCTGATGTTGATGGAGACAGATATTTTGGCATCCAGTCCTTCAGTGTTCG
CCTGGGCCAAGAAAAAGTGTTTTGGTTTTGCATCAAGTTGTTGTTAACTGCATATGCTACAGCT
CTATTGGTGGGAGCTTCTACATCAACCATATATCAGAAGATTGTGACTGTGCTTGGCCATGGCT
TGCTTGCTTCCATTCTTTGGTTTCGGGCACAGTCTATTGATCTTCAGAACAAGGCATCCATAAC
CTCATTCTACATGTTCATTTGGAAGCTGTTTTATGCCGAGTATTTCCTTATCCCTTTCATGCAC
TGAAACCCAGCTTTCTTGTAC

STOP

> pB7WG2D.1+ *HighM HGGT*

START

TTTGTACAAAAAAGCAGGCTATATGGCAAAAGAAGAGTTTATCAATACAAATATTCGCAAATCA
GAATACTTCTCTCCTCTCCTCGCCGTGCTCCTCCTCTGTTCTTCTTCTNCTCTCTCTAAATCAC
AATACTTCTCTTCTCTCCTCTCCTTGCCGTACTCCTCCTCTGTTCTTCATCTTCTCTCTTGGGA
AATGCTGTCCAACCAGGCTGTTTCAGTCTCCCGTTTTACCAAGTGTTGGCTCCCTCTAAGAGAT
GGGACAAGAGGAAGAGGGCCCATTACTGGTACTCAAGGGAGAGGTGGAATTTGGAAAGTAGAAA
ACTTGGAAGTCCAAGGAACAAGATGTAAATCCCCTAAATTCAAAGATATAGTCTATGATCTAAA
GCATAAAGAGAGGCCATCAATGCATTATAGACGACTTACAAAAGGTTGCATTTCTGCTGCTTCT
GAGCTTGCATATGTACCCGAACCAACGGATAATCAGTCACAAGGTCTATGGACTGCTGTTTCTA
AAAAGTTAGATGCTTTCTATCGGTTTTCTCGTCCCCACACAGTAATAGGCACTATCGTAGGCAT
AGTATCAGTCTCTCTTCTTCCAGTACAAAGTCTCGCTGATTTTTCTCCAACATATTTTATNGGA
CTATTGAAGGCATTGGTTCCAGCTGTATGCATGAACATCTATGTTGTGGGCCTGAACCAGCTAT
TTGACATAGAAATAGATAAGGTTAATAAACCTAGGCTTCCACTTGCTTCAGGCGAGTTCTCTTT
GGGAACAGGAATTGTGTTGGTATTTGCCGCTGCTATCATGAGCTTTACAATGGGGTTGCAGTCC
AAGTCCCCTCCCCTATTAGGTGCCTTGCTCATCAGCTGTTTTCTTGGAAGTGCATATTCCATCA
ATATTCCCTTCTTTAGATGGAAACAGCATGCCTTTCTTGCTGCATCTTGCATCTTGTGTGTGAG
GGCCGTTGTAGTCCAGCTAGCTTTTTTCATTCACATGCAGAGATATGTACTAGGAAGACCAACA
GTGTTGACAAAACCAGTGATATTTGCAACAGCTTTCATGTGTTTTTTCTCAGCTGTTATTGCTC
TGTTCAAGGATATACCTGATGTTGATGGAGACAGATATTTTGGCATCCAGTCCTTCAGTGTTCG
CCTGGGCCAAGAAAAAGTGTTTTGGTTTTGCATCAAGTTGTTGTTAACTGCATATGCTACAGCT
CTATTGGTGGGAGCTTCTACATCAACCATATATCAGAAGATTGTGACTGTGCTTGGCCATGGCT
TGCTTGCTTCCATTCTTTGGTTTCGGGCACAGTCTATTGATCTTCAGAACAAGGCATCCATAAC
CTCATTCTACATGTTCATTTGGAAGCTGTTTTATGCCGAGTATTTCCTTATCCCTTTCATGCAC
TGAAACCCAGCTTTCTTGTAC

STOP

> pB7WG2D.1+ *Low HGGT*

START

TTTGTACAAAAAAGCAGGCTATATGGCAAAAGAAGAGTTTATCAATACAAATATTCGCAAATCA
GAATACTTCTCTCCTCTCCTCGCCGTGCTCCTCCTCTGTTCTTCTTCTCTCTCTCTAAATCAC
AATACTTCTCTTCTCTCCTCTCCTTGCCGTACTCCTCCTCTGTTCTTCATCTTCTCTCTTGGGA
AATGCTGTCCAACCAGGCTGTTTCAGTCTCCCGTTTTACCAAGTGTTGGCTCCCTCTAAGAGAT
GGGACAAGAGGAAGAGGGCCCATTACTGGTACTCAAGGGAGAGGTGGAATTTGGAAAGTAGAAA
ACTTGGAAGTCCAAGGAACAAGATGTAAATCCCCTAAATTCAAAGATATAGTCTATGATCTAAA
GCATAAAGAGAGGCCATCAATGCATTATAGACGACTTACAAAAGGTTGCATTTCTGCTGCTTCT
GAGCTTGCATATGTACCCGAACCAACGGATAATCAGTCACAAGGTCTATGGACTGCTGTTTCTA
AAAAGTTAGATGCTTTCTATCGGTTTTCTCGTCCCCACACAGTAATAGGCACTATCGTAGGCAT
AGTATCAGTCTCTCTTCTTCCAGTACAAAGTCTCGCTGATTTTTCTCCAACATATTTTATTGGA
CTATTGAAGGCATTGGTTCCAGCTGTATGCATGAACATCTATGTTGTGGGCCTGAACCAGCTAT
TTGACATAGAAATAGATAAGGTTAATAAACCTAGGCTTCCACTTGCTTCAGGCGAGTTCTCTTT
GGGAACAGGAATTGTGTTGGTATTTGCCGCTGCTATCATGAGCTTTACAATGGGGTTGCAGTCC
AAGTCCCCTCCCCTATTAGGTGCCTTGCTCATCAGCTGTTTTCTTGGAAGTGCATATTCCATCA
ATATTCCCTTCTTTAGATGGAAACAGCATGCCTTTCTTGCTGCATCTTGCATCTTGTGTGTGAG
GGCCGTTGTAGTCCAGCTAGCTTTTTTCATTCACATGCAGAGATATGTACTAGGAAGACCAACA
GTGTTGACAAAACCAGTGATATTTGCAACAGCTTTCATGTGTTTTTTCTCAGCTGTTATTGCTC
TGTTCAAGGATATACCTGATGTTGATGGAGACAGATATTTTGGCATCCAGTCCTTCAGTGTTCG
CCTGGGCCAAGAAAAAGTGTTTTGGTTTTGCATCAAGTTGTTGTTAACTGCATATGCTACAGCT
CTATTGGTGGGAGCTTCTACATCAACCATATATCAGAAGATTGTGACTGTGCTTGGCCATGGCT
TGCTTGCTTCCATTCTTTGGTTTCGGGCACAGTCTATTGATCTTCAGAACAAGGCATCCATAAC
CTCATTCTACATGTTCATTTGGAAGCTGTTTTATGCCGAGTATTTCCTTATCCCTTTCATGCAC
TGAAACCCAGCTTTCTTGTAC

STOP

DISCUSSION

Double site-directed mutagenesis was carried out here in order to ascertain the functions of two important sequence variants in HGGT gene one of the most important gene that committed the first step for vitamin E biosynthesis in *Elaeis guineensis* (Yang et al., 2011 Atanassov et al., 2009). The first mutation leads to change in amino acid from proline to serine which occurs by substitution of cytosine (C) to thymine (T) at position 91 of the cDNA (Jensen and Weilguny, 2005). The second mutation leads to change in the amino acid from methionine to isoleucine when guanine (G) is substituted with adenine (A) at position 615 of the cDNA. This was accomplished by introducing the desired nucleotide change into specific oligonucleotide primers (Kaur and Sharma, 2006) and used it in PCR-mediated overlap extension method (Heckman and Pease, 2007). The entire cDNA was broken into two fragments during each substitution mutation. The first PCRs with primers A+B and C+D produce AB (101 bp long) and CD (1288 bp long) fragments and AB (629 bp long) and CD (760 bp long) fragments at cDNA sequence positions 91 and 615 respectively with primer B and C introducing the desired mutation. In this experiment the internal primers are completely complementary with one another. The base substitution for mutation is located between 5 to 10 nucleotide from 5'end of the forward primer because internal primers must not only contain the desired mutation, but also must create overlapping nucleotide sequences, so that when the strands of products AB and CD are denatured during the second PCR, strands with 3' complementary ends created by mutagenic primers b and c will easily anneal (Zeng et al, 2017).

Precise and careful design of these primers (especially b and c) is critical to the success of this procedure.

Another PCR-mediated method is used to extend the overlapping fragments containing the site-directed mutagenesis (substitutions) using two flanking master primers (A and D) that mark the 5'ends of both strands, this amplified one of those hybridization products (sense strand from AB and antisense strand from CD) to generate the predominant AD product with the intended mutation (Heckman and Pease, 2007). The high efficiency and relative simplicity of this PCR-mediated overlap extension technique make it a valuable strategy for generating a range of desired gene modifications (Liang et al., 2012).

Primer set AD was modified into *AttB*-set of gene specific primers and used to amplified all the mutated products generated by site-directed mutagenesis by PCR and cloned into pDONR^{TM}Zeo as described in the manufacturer's protocol. The mutant sequences was verified by colony PCR screening and sequencing (Kramer et al., 2003) which is carried out both before and after ligation into destination vector pB7WG2D.1 (ref) using LR Clonase (Invitrogen). This confirmed that the mutant and original type cDNAs of HGGT gene from *E. guineensis* can be inserted into expression vector to transform bacterial cells by gateway cloning to generate large quantity of cDNAs for proper analysis.

CONCLUSION

This research indicates that coding regions of *E. guineensis* genes can directly be mutated by site-directed mutagenesis and amplified using PCR overlap extension without necessarily cloning the gene of interest into any plasmid before been use for mutagenesis. This new approach was successfully used to generate three different cDNA sequences molecules (*91M HGGT, 615M HGGT* and *2M HGGT*) that are 99.99 % homologous to original wild type cDNA (*Low HGGT*) of *E. guineensis* thereby saving money and time on chemically synthesized oligos and avoiding any chemical changes that may arise from artificially synthesize cDNA that can alter the structural elements which are critical to the gene biological functions.

ACKNOWLEDGEMENT

A sincere thank goes to Prof. Datin Dr. Siti Nor Akmar Abdullah of the Department of Agrobiotechnology, Faculty of Agriculture, University Putra Malaysia for providing enabling environment, fund and facilities in her Molecular Biotechnology Laboratory at Institute of Tropical Agriculture (ITA) UPM to carry out this noble research successfully.

REFERENCES

Atanassov, I.I., Etchells, J.P., Turner, S.R. (2009). A simple, flexible and efficient PCR-fusion/Gateway cloning procedure for gene fusion, site-directed mutagenesis, short sequence insertion and domain deletions and swaps. Plants Methods, 5:14

Chiu, J., March, P.E., Lee, R., Tillett, D. (2004) Site-directed , Ligase-Independent Mutagenesis (SLIM): a single-tube methodology approaching 100 % efficiency in 4 h. *Nucleic Acids Research*, 32: 174-179.

Heckman, K. L. and Pease, L. R. (2007). Gene splicing and mutagenesis by PCR-driven overlap extension. *Nat Protoc*, *2*(4), 924–932. http://doi.org/10.1038/nprot.2007.132

Hilson, P. I. I., Etchells, J. P., Turner, S. R., Hilson, P., Dafny-Yelin, M., Terpe, K. (2006). Cloned sequence repertoires forsmall- and large-scale biology. *Trends in Plant Science*, *11*(3), 133–141. http://doi.org/10.1016/j.tplants.2006.01.006

Jensen, P. H and Weilguny, D (2005) Combination primer polymerase chain reaction for multi-site mutagenesis of close proximity sites. *Journal Biomolecule and Technology*. 16: pp. 336–340

Karimi, M., Inzé, D. and Depicker, A. (2002) Gateway vectors for *Agrobacterium*-mediated plant transformation. *Trends in Plant Science*, 7: 193–195.

Kaur, J. and Sharma, R. (2006). Directed Evolution: An Approach to Engineer Enzymes. *Critical Reviews in Biotechnology*, *26*(3), 165–199. http://doi.org/10.1080/07388550600851423

Kiryu, I., Nishioka, T., Yuasa, K., Kurita, J., Shimahara, Y., Ototake, M. and Oseko, N. (2014) Rapid and Simple Detection Method of ldquo; Candidatus Xenohaliotis californiensis rdquo; Using Fecal PCR in Abalone Haliotis discus discus and H. gigantea. *Fish Pathology*, *49*(2), 41–48. http://doi.org/10.3147/jsfp.49.41

Liang, X., Peng, L., Li, K., Peterson, T. and Katzen, F. (2012). A method for multi-site-directed mutagenesis based on homologous recombination. *Analytical Biochemistry* (427).

Liu, J., Huang, S., Sun, M., Liu, S., Liu, Y., Wang, W., Zhang, X., Wang, H., Hua, W. (2012) An improved allele-specific PCR primer design method for SNP marker analysis and its application. *Plant Methods*. 8: 34.

McCullum, E. O., Williams, B. A. R., Zhang, J. and Chaput, J. C. (2010). Random mutagenesis by error-prone PCR. *Methods in Molecular Biology*, *634*, 103–109. http://doi.org/10.1007/978-1-60761-652-8_7

Urban, A. (1997). A rapid and efficient method for site-directed mutagenesis using one- step overlap extension PCR. *Nucleic Acids Research*, 25: 2227–2228.

Yang, W., Cahoon, R.E., Hunter, S.C., Zhang, C., Han, J., Borgschulte, T., Cahoon, E.B. (2011) Vitamin E biosynthesis : functional characterization of the monocot *homogentisate geranylgeranyl transferase*. *The Plant Journal*, 65: 206–217

Zeng, F., Zhang, Y., Zhang, Z., Malik, A.A., Lin, Y., Zeng, F. (2017. Multiple-site fragment deletion , insertion and substitution mutagenesis by modified overlap extension PCR 2818. *Biotechnology & Biotechnological Equipment*, 31: 339–348